Abstract Number: 4561

Anti-tumor effects of duramycin-IgG conjugate

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Duramycin is a nonadecapeptide antibiotic obtained from Streptoverticillium Cinnamomeus which recognizes strictly the structure of phosphatidylethanolamine (PE) and forms an equimolar complex with the phospholipids on biological membranes. The molecule has a high amount of cross-linking, including thioester linkage of lanthionine and an imino bridge of lysinoalanine which may account for its unique stability. Duramycin forms aqueous pores in the membranes after its binding to PE. It shows hemolytic activity against erythrocytes of various animal species.

We hypothesized that PE, like phosphatidylserine, would become exposed on tumor vasculature as a result of exposure to stress conditions in the tumor microenvironment. It is known that PE as well as PS is exposed on the cell surface during the early stages of apoptosis, resulting in the loss of asymmetric distribution of phospholipids in the plasma membrane bilayer.

To detect cell surface phosphatidylethanolamine, we generated duramycin-IgG conjugate which retains its PE binding capacity while losing its hemolytic effect. The mouse IgG2a was used as the carrier protein to bring host defenses to act against cells that bind duramycin-IgG. Duramycin mouse IgG2a conjugate bound effectively to the apoptotic cell surface, but not to non-apoptotic cells. The binding to apoptotic cells was not uniform and the intense staining was observed on surface blebs of apoptotic cells. The conjugate enhanced the phagocytosis of apoptotic cells by murine bone-marrow derived macrophages in vitro. After intravenous injection into mice bearing solid tumors, duramycin-IgG localized to tumor endothelium. Between 15% and 40% of tumor blood vessels were stained. Necrotic regions of tumors were also stained. In contrast, none of the blood vessels in normal tissues were stained. The duramycin-IgG conjugate inhibited the growth of syngeneic MethA tumors in BALB/c mice. No toxicity was observed in mice treated with the conjugate. Histological examinations of mouse MethA tumors from duramycin-IgG treated mice revealed large numbers of macrophages in the tumor interstitium and attachment to the tumor endothelium.

Conclusion:

Duramycin-IgG is a promising reagent for clinical studies of cancer patients.

Exhibit H

Anti-tumor Effects of Duramycin-IgG Conjugate Targeted to Phosphatidylethanolamine on Tumor Blood Vessels.

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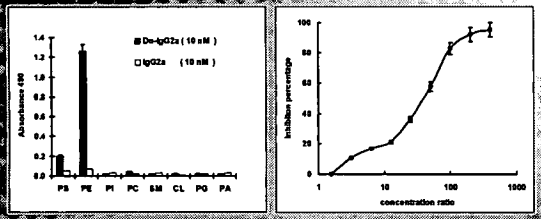
INTRODUCTION

Duramycin is a nonadecapeptide antibiotic which recognizes strictly the structure of phosphatidylethanolamine (PE). PE and phosphatidylserine (PS) are largely absent from the external leaflet of plasma membrane of resting mammalian cells. PE and PS, become exposed on the cell surface during events such as apoptosis, injury and malignant transformation, that result in the loss of phospholipids asymmetry in the plasma membrane. We hypothesized that PE, like PS, would become exposed on the external surface of tumor vasculature as a result of exposure to stress conditions in the tumor microenvironment. To test this hypothesis, we generated a duramycin-IgG conjugate which retained its PE binding capacity while losing the hemolytic effect that is normally associated with duramycin. We investigated the ability of duramycin-IgG to home selectively to PE on tumor vessels and exert an anti-tumor effect.

PURPOSE

- To generate duramycin mouse IgG2a conjugate and characterize the PE binding ability.
- To investigate duramycin-IgG2a binding to PE positive cells *in vitro*.
- To investigate duramycin-IgG2a localization to PE positive tumor vessels *in vivo*.
- To investigate the anti-tumor effect of duramycin-IgG2a.
- To determine whether the mechanism of the anti-tumor effect might be mediated through macrophages binding to tumor vessels.

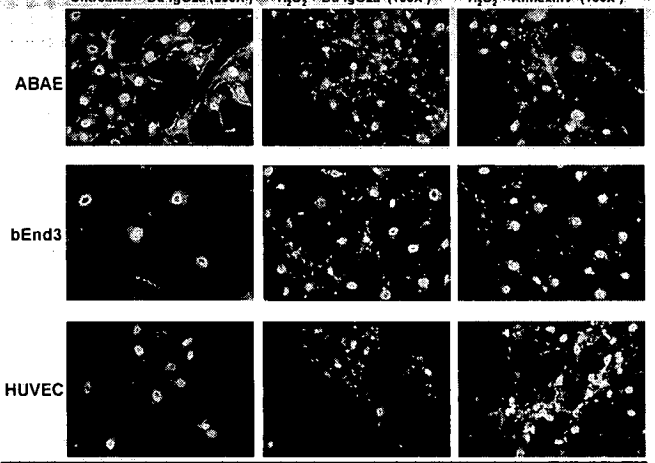
Fig 1. Duramycin-IgG2a binding to PE was specific and can be blocked by duramycin.



Phospholipids dissolved in *n*-hexane were coated on 96-well plates. After blocking, Duramycin-IgG2a at concentration ranging from 10 nM to 0.005 nM was added and incubated at 37 □ for 1 h. After washing, the bound duramycin-IgG2a conjugate was detected using peroxidase labeled goat anti-mouse IgG antibody. Duramycin-IgG2a conjugate recognized PE only. It had no detectable binding to other common phospholipids (left). The binding specificity of duramycin-IgG2a to PE was further determined using a competitive ELISA method in the excess amount of duramycin (right).

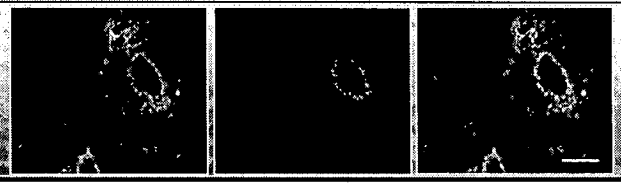
RESULTS:

Fig 2. Duramycin-IgG2a binding to PE on the surface of H_2O_2 treated cell membrane.



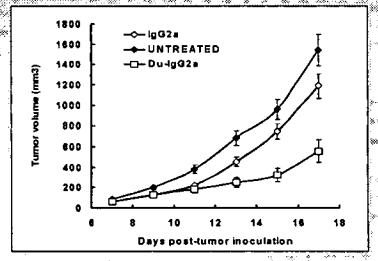
Duramycin-IgG2a bound to all three endothelial cell species. To mimic the tumor environment stress condition, endothelial cells were treated with 200 μM hydrogen peroxide for 1 h. Live cells were incubated with duramycin-IgG2a for 1 h, and then fixed with 4% paraformaldehyde. Exposed PE was detected with duramycin-IgG2a (visualized by green fluorescence from secondary anti-mouse IgG-FITC). Biotin-annexin V was used as positive control. Cytoskeleton was detected with Texas-red labeled phalloidin. Nuclei were stained with DAPI.

Fig 3. Duramycin-IgG2a localizes to blood vessels of MDA-MB-435 tumors.



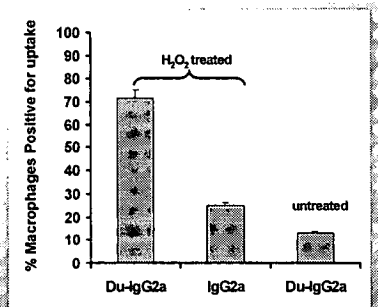
SCID mice bearing orthotopic MDA-MB-435 tumors were injected i.v. 100 μg duramycin-IgG2a. 4 h later mice were exsanguinated and perfused. 10 μm cryosections of tumor and major organs were prepared. Localized duramycin-IgG2a was visualized as green color. Endothelium was visualized as red color. Tumor sections derived from mice injected with the same amount of IgG2a served as negative controls. No localization was found in normal blood vessels. Merged picture illustrated that duramycin-IgG2a localized to tumor endothelium (yellow color). Scale bar represents 100 μm.

Fig 4. Duramycin-IgG2a inhibits the growth of MethA tumor.



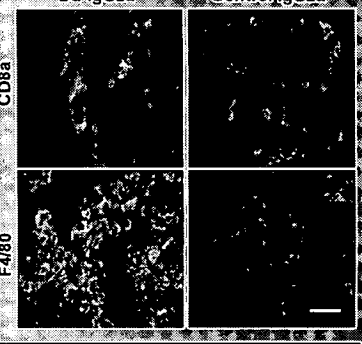
Groups of BALB/c mice with established Meth A fibrosarcoma were injected i.p. either with 100 μg of duramycin-IgG2a (n = 10) or 100 μg of IgG2a (n = 10) Q.O.D. for 2 weeks. Tumor growth was measured every other day. Mean tumor volumes are shown. Bars, SE. Duramycin-IgG2a significantly retarded syngeneic Meth A tumor growth ($P < 0.05$).

Fig 5. Duramycin-IgG2a enhances phagocytosis of apoptotic cells *in vitro*.



Phagocytosis of apoptotic HL-60 cells by bone-marrow derived macrophages from BALB/c mice was enhanced by duramycin-IgG2a. Data represent means of three experiments ± SE. For the experiments shown, 13% macrophages phagocytosed normal HL-60 cells, 72% phagocytosed duramycin-IgG2a treated apoptotic HL-60 cells, and 23% phagocytosed IgG2a treated apoptotic HL-60 cells.

Fig 6. Duramycin-IgG2a induces macrophage and lymphocyte infiltration into tumors



Mouse macrophages and lymphocytes were detected with rat anti-mouse F4/80 and CD8a Abs visualized as green color. Blood vessels were stained with hamster anti-mouse CD31 Ab visualized as red color. Upper left showed CD8a positive lymphocytes were enriched surrounding tumor endothelium. Lower left showed F4/80 positive macrophage number was increased in duramycin-IgG2a treated tumor-bearing mice. Images on the right side stained with identical Ab were from tumor sections of control Ab treated mice. Scale bar represents 100 μm.

CONCLUSION:

- PE is exposed on the surface of tumor blood vessels.
- Duramycin-IgG2a can bind PE positive cells *in vitro* and PE positive tumor endothelium *in vivo*.
- Duramycin-IgG2a inhibited syngeneic MethA tumor growth.
- The anti-tumor effect of duramycin-IgG2a may be due to host immune cells infiltration into tumor and attack of tumor endothelium.

ACKNOLEDGEMENT

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